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13. ABSTRACT (Maximum 200 Words)

This program was designed to recruit qualified undergraduates with an interest in research, and to provide them with a highly interactive program that integrated the unique expertise available in our laboratories in the Life Sciences Division of the E.O. Lawrence Berkeley National Laboratory and the Department of Cell & Molecular Biology at the University of California, Berkeley, and to guide them towards developing an interest in investigating the underlying mechanisms involved in the development of breast cancer. Trainees in the first year of the program benefited from working in a dynamic environment that investigates issues at the forefront of breast cancer research. The students chose from projects investigating the effects of hormones on rodent mammary glands, working with human breast cells in culture, and dissecting transcription functional alterations in yeast. In the first category, students studied the current literature on hormones and breast cancer and worked with a postdoctoral fellow or a staff member conducting research in breast cancer in rodents. The research in the latter two categories of projects involved studies of processes known to function differently in normal and malignant breast cells. Projects were designed with a goal towards cohesive research objectives that were meaningful, educational, and attainable.

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SUMMARY

This research report describes the second year of the BCRT undergraduate research training program in breast cancer. The goal of this project is to provide undergraduate trainees with exposure to areas of breast cancer research that focus on the role of microenvironment in mammary gland biology and in the development of neoplasia. Trainees in this project benefited from working in a program that investigates the intersection of hormones, growth factors, and extracellular matrix (ECM) signaling and remodeling during mammary gland morphogenesis, differentiation, and carcinogenesis. The program was advertised through several undergraduate research forums on the UC Berkeley campus, and more than four dozen applications were received. From these, eight applicants were selected to represent a balance of interests and approaches, with broad levels of expertise ranging from laboratory novices to students with many years of laboratory experience. During the research portion of the program, undergraduate trainees had frequent interaction with mentors and with advanced postdoctoral fellows, and reports were presented in organized, biweekly meetings structured to reflect the organization of a research paper. At the first meeting, the students presented the introduction to their research project; at the second, the materials and methods; at the third, the results. For the final meeting of the program, the students presented their work in complete form, complete with conclusions and interpretations. While the success at obtaining experimental results within the allotted time of the research program varied, all the participants (both student and preceptors) agreed that the overall experience was successful.

BODY

In this section, research accomplishments of individual undergraduates will be summarized, with an emphasis on the forward direction of the projects.

Irving Salmeron
Mary Helen Barcellos-Hoff Lab
Supervisor: Helen Oketch
Project Title: Effects of Irradiated Stroma on Tumorigenesis of p53 null Mouse Mammary Epithelium

ABSTRACT

At the cellular level, the acquisition of DNA mutations can lead to cells escaping cell cycle regulation mechanisms and eventually becoming cancerous. However, the environment of the cells, for example the stroma for epithelial cells in the mammary gland, is equally important in maintaining normal function of the cells.

The hypothesis is that ionizing radiation (IR) modifies the stroma providing it with the ability to accelerate/promote tumorigenesis in epithelial cells with which it comes in contact. The study tested this hypothesis by investigating the effect of irradiated stroma on the tumor forming ability of two tumorigenic mammary outgrowth

lines, one lacking the p53 gene and the other wild-type for the p53 gene. Although at the time of writing this report the experiments were still in progress, we had sufficient data to determine that tumors from the IR stroma in both groups had a longer latency than those from the sham stroma. Also the growth rate of tumors was independent of either treatment of the host animas or fragment transplanted.

Although we can not make final conclusions because these experiments are still in progress, the results up to this point indicate that the effect of the irradiated stroma here is inconsistent with previous findings that showed that it accelerated tumorigenesis. This indicates that the process of tumorigenesis in transplanted fragments is dependent on a two way interaction between the IR stroma and the transplanted fragments. We propose that the effects of the irradiated stroma are dual such that it can either accelerate or inhibit tumorigenesis depending on the whether the fragments transplanted are initiated into the tumorigenic process and have progressed or not. Thus we propose that cells that have been initiated into the tumorigenesis process are accelerated by the irradiated stroma environment, while those that have not been initiated are deleted by apoptotic signals or arrested by cell cycle signals.

INTRODUCTION

Control of individual cell function lies not only within the cell but is also a result of its microenvironment, cell-cell interactions, extracellular matrix, growth factors and hormones (1). The ECM is normally programmed to send signals to the cells which would suppress genomic mutations when they appear by sending apoptotic signals. However, as exposure to low doses of ionizing radiation intensifies, this defense program becomes corrupted and the wrong signals get transmitted (2). Previous studies at the Barcellos-Hoff lab have shown that ionizing radiation alters the composition of tissue microenvironment by rapidly affecting cytokine production and activities, extracellular matrix composition, and expression of receptors that mediate cell-cell interactions (3).

Studies have shown that when unirradiated non-tumorigenic mammary epithelial COMMA-D cells were transplanted to the cleared fat pad (CFP) of mice that had been exposed to ionizing irradiation, tumors formed rapidly and at a high frequency (4). The same cells transplanted to the CFP of non-irradiated mice formed normal mammary glands, indicating that irradiation of neighboring non-target cells (the stroma in this case) can create a cancer-promoting environment.

To address the question of whether irradiation of the stroma was accelerating mammary tumorigenesis, unirradiated preneoplastic p53*null* mammary fragments were transplanted to irradiated and sham (control group) cleared fat pad of syngeneic Balb/c mice. The p53*null* fragments usually develop mammary tumors when transplanted to sham mice at about one year of age (5). The results showed neither increased tumor incidence or decreased tumor latency in irradiated mice, but surprisingly showed that animals irradiated with dosages lower than 4Gy total body irradiation developed fewer tumors than those exposed to higher dosages indicating some protective effect of lower dosages of IR and a higher tumor growth rate than in tumors that developed in the sham stroma (6).

The present study was designed to determine whether the above results would be reproducible with preneoplastic mammary epithelial outgrowth lines whose tumorigenic

characteristics are already well established. The OG line fragments differ from other transplanted fragments in their rate of growth, stable phenotype and their ability to be transplanted infinitely without diminishing tumorigenic potential of the cells (7). We used two outgrowth lines a p53*null* outgrowth line and a wildtype outgrowth line. The aim was firstly to determine whether the inverse dose response and protective effect of low dose irradiation that was observed with the p53*null* fragments is reproducible with the p53*null* OG line (PN1a) that was obtained from Dan Medina. Secondly to determine whether similar results would be obtained with a p53 wild type OG line (TM2H).

MATERIALS AND METHODS

Animals

All experiments were conducted with institutional review and approval. Animals were euthanized by CO2 inhalation and cervical dislocation at the indicated times in accordance with AAALAC guidelines. Animals were housed at the LBNL animal facility under a controlled environment.

Three weeks old female Balb/c host mice, weighing 9-12 grams were purchased from B&K or Simonson. Mammary epithelium fragments of outgrowth lines were obtained from outgrowth of samples that were kindly donated by Dan Medina of the Baylor College of Medicine which were transplanted to the CFP of wild type Balb/c mice in the Barcellos-Hoff lab. For the experiment mice were divided into treatments groups as shown in Table 1 below:

Treatment Groups	Fragments transplanted and number of animals in each group	
	PN1a (p53null)	TM2H (wt)
IR	30	30
Sham	8	8
Total	**36**	**36**

* Sham: not irradiated (control group)
* Ionizing radiation dosages: 01, 0.5, 2.0 and 4 [Gy]

Clearing of Fat Pads (CFP)

At 3 weeks of age, the inguinal gland was cleared as follows: an incision was made to expose the inguinal gland then using tweezers, the nipple is lifted and all its attachments to the skin are cut and removed all the way up to the lymph node (including the lymph node) with scissors and thermocautery.

Transplantation

Three days before transplantation, the mice were irradiated whole body with 4 Gy using ^{60}Co source of radiation. The CFP was transplanted with fragments of the OG lines on both inguinal glands. An extra mouse in the sham group was sacrificed at three weeks after transplantation in order to confirm outgrowth of the mammary transplants.

Animal Observations

Overall health of mice was regularly monitored three times a week noting the weight, size, aloepocia, and eye problems (animal observations and care may take as long as several months to a year depending on the type of transplanted fragment). Animals were gently palpated three times a week for tumors and then detected tumors were measured until they attained a size of 1x1cm. This information was used to determine incidence, latency and rate of tumor growth. All animals that developed tumors were included in the study. However animals were excluded from the study if they were found critically injured or developed other severe health problem that necessitated their termination.

Collecting Tumors

Animals were euthanized by C02 inhalation and cervical dislocation. The tumor was preserved as follows: ¼ in liquid nitrogen for protein, RNA and DNA studies for aCGH, Western Blot etc, ¼ embedded in OCT (Optimal Cutting Temperature) for immunohistochemistry, ¼ as fragments of 2mmx2mm stored at -80degrees for transplantation in future experiments, and¼ in PF (4% Paraformaldehyde) for histology studies. The 4th MG (tumor-free) was collected in ½ in OCT and ½ prepared as wholemount. The 3rd MG were also processed for wholemount analysis to check for normal mammary development.

RESULTS

In Figure 1a, 1b) The y-axis of the OG line graphs represent the number of tumor-free fat pads. Figure 1c) shows the latency curves of a previous transplant experiment. In both experiments it took longer for the tumors in the 4Gy irradiated mice to form than in the sham animals.

Figure 1a: A0403PN1a Tumor Free Curve

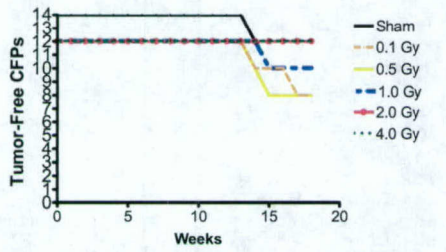

Figure 1b: A0403TM2H Tumor Free Curve

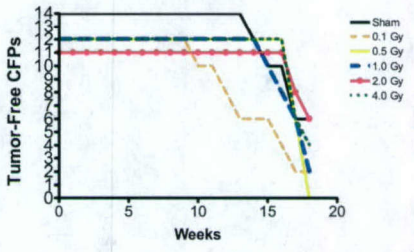

Figure 1c:

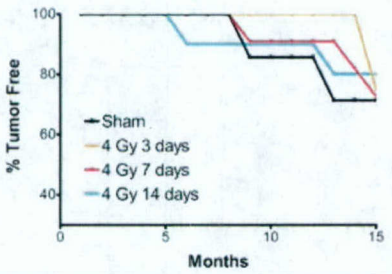

Figure 1a, b: Tumor free curves showing duration in weeks when fat pads were free of tumors. Figure 1c: The tumor free curve for a previous experiment; note that duration is in months.

At the intermediate dosages no conclusions can be made because the experiment is still ongoing. Also, the latency for the wildtype line was much shorter than the p53*null* line.

In Figure 2a, 2b) the y-axis of the growth rate graphs represents the average growth rate of the fat pads that formed tumors for that particular group. The number within the column bars refers to the number of fat pads that had tumors.

Figure 2a:

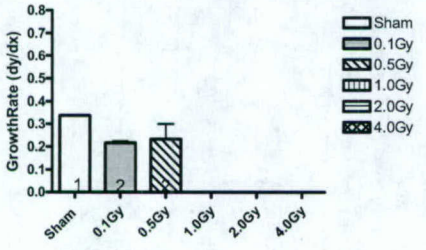

Figure 2b:

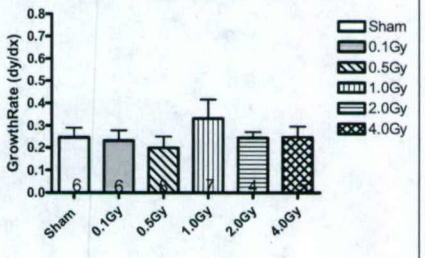

Figure 2a, b: Tumor growth rate graphs was calculated by taking the average of tumor growth rate of tumors that reached endpoint (1x1cm) per group. Note the small number of tumors making up the data for Figure 2a.

PN1a (p53*null*) OG line had a longer latency and therefore there were fewer tumors to be plotted by the time this analysis was done. Consequently not much can be said about this experiment. From the TM2H (wildtype) OG line there was no evidence that IR stroma increased tumor growth rate as was previously observed in the transplants with unirradiated preneoplastic p53*null* mammary fragments. The 2.0Gy group for the TM2H line had a large standard deviation which once taken into account, allows the conclusion that IR had no effect on tumor growth for either of the OG lines.

DISCUSSION

We used OG line cell fragment transplants to see if we could reproduce the observations from previous experiments as well as to compare and contrast tumorigenesis of the two outgrowth lines. The result showed longer latency period for the tumors arising in the irradiated stroma compared to the sham stroma for both OG lines. The inverse dose response and protective effect of low dose irradiation that was previously observed with p53*null* fragments was not evident in this experiment. Also the accelerated growth of tumors in the irradiated stroma as previously observed was not apparent with the OG lines. One possible explanation for the previously observed accelerated growth of p53*null* tumors in the irradiated stroma may be related to the age of the host mice that were much older than the host mice used in this experiment.

A possible reason as to why the wildtype line formed tumors much faster compared to the p53*null* OG line could be probably due to the nature of the OG line. The wildtype outgrowth line is reported to form tumors three months after transplant which is consistent with our results. This study was a follow up to previous experiments that had used p53*null* mammary gland fragments from p53*null* mice. It took about one year for tumors to form. In the current experiments we are switching to OG lines that are well characterized phenotypically and have a much shorter tumor latency period. The focus was the role of irradiated stroma and observations were made in reference to it. This study has shown that the current and previous observations might be more results of the type of fragment used rather than specific phenomena of the stromal environment resulting from the irradiation.

CONCLUSION

The study of the microenvironment in tumorigenesis is necessary in order to understand the tumorigenesis process at the tissue level and to define the interactions involved therein. Studies have shown that regulation of tissue by the microenvironment is what the aberrant cells must surmount if they are to progress to form tumors. This study has been designed to understand the processes involved in the communication between the epithelial cells and the stroma.

From the findings of this study, it can be concluded that the type of "seed" (epithelium fragments) used in the "soil" (stromal environment), plays a vital role in the type of observations that have and are being made in terms of tumorigenesis. To truly understand the role of the microenvironment in the tumorigenesis process the quest lies not in soley understanding how it responds but also just as importantly in understanding what it responds to.

ACKNOWLEDGEMENTS

I am grateful to the Breast Cancer Research Training Fellowship and to Dr Mary Helen Barcellos-Hoff for this opportunity that I was given to contribute to research efforts in breast cancer. The guidance and kindness of everyone in the Barcellos-Hoff Lab, especially that of my mentor Dr. Hellen Oketch-Rabah, is greatly appreciated and cherished.

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Anna Guan
Caroline Kane Lab
Supervisor: Sarah Archer-Evans
Project Title: Identification of Genes Regulated by Transcription Factor IIs in *Saccharomyces Cerevisiae* Treated with Hydrogen Peroxide

Abstract

The regulation of transcription is essential to human health. The process of transcription involves promoter binding and transcript initiation, promoter escape, transcript elongation, and termination. Recently, there has been a great focus on the elongation step of RNA synthesis because many genes controlling essential cellular processes such as c-myc oncogenes are regulated during the elongation phase of transcription. The c-*myc* oncogene plays a central role in cell proliferation and apoptosis, and is generally overexpressed in human cancer. The transcriptional machinery is highly conserved between prokaryotes and eukaryotes. Among the many factors that regulate transcription is the transcription factor IIS (TFIIS) found in eukaryotes and its prokaryotic counterpart GreB factor. Lesions on DNA caused by oxidative stress can block RNA polymerase II from transcribing. TFIIS protein promotes the RNA polymerase II to read-through this type of block by stimulating the polymerase to cleave the nascent transcript. Hydrogen peroxide causes oxidative stress in *Saccharomyces cerevisiae*. Previous work in the Kane laboratory has shown that TFIIS deleted yeasts have a higher percent survival rate than wild type yeasts in the presence of hydrogen peroxide suggesting that TFIIS plays an important role in yeasts under oxidative stress.

The objective of this project is to identify genes regulated by TFIIS *in vivo* via a global genomic approach using DNA microarrays. The arrays are using cDNAs prepared from mRNAs obtained from hydrogen peroxide treated cells containing the TFIIS gene and ones lacking TFIIS. Cy3 and Cy5 are used to label the cDNAs from the two types of cells. Hybridizations of both types of cDNAs simultaneously allow a direct comparison by ratios of intensity of the differentially fluorescing signals.

Preliminary results show that in TFIIS deleted cells relative to wild type TFIIS cells, seven proteins involved in metabolic pathways are upregulated at least two fold and two proteins involved in oxidative stress response are downregulated at least two fold when these types of cells are treated with peroxide. Our findings suggest that these proteins might be regulated by TFIIS in *Saccharomyces cerevisiae* during oxidative stress. These results will add to our understanding of transcription regulation of oncogenes,

whom overexpression lead to breast cancer; hence, these results will aid in the effort to find a cure for breast cancer.

Introduction

Transcription is the process in which RNA is synthesized by RNA polymerase using a DNA template. The RNA is a mobile copy of the genetic information encoded by DNA and conveys the genetic information contained in the DNA to the ribosome to manufacture the necessary proteins for the survival of the cell. Since transcription is connected with all other cellular processes, defects in this process have severe human health consequences, which could lead to tumorigenesis. Transcription comprises of four distinct stages: promoter binding and initiation, promoter escape, transcript elongation, and termination. Recently, there has been great focus on the elongation step of RNA synthesis. Among the different genes regulated during elongation are genes involved in cell cycle, development, and differentiation such as the c-*myc* oncogene[1]. The c-myc oncogene plays an important role in cell proliferation and apoptosis and disregulation has been shown to be a major aspect in tumorigenesis [2].

The transcriptional machinery is highly conserved between prokaryotes and eukaryotes [3]. Numerous proteins assist RNA polymerase with the process of transcription, but only eukaryotic transcription factor TFIIS and its prokaryotic homolog GreB can restart an arrested RNA polymerase [1,4]. An arrest occurs when the RNA polymerase encounters a lesion causing the 3'-end of the nascent transcript to become misaligned with the catalytic site of the polymerase [5]. Once an RNA polymerase arrests, it binds tightly to the DNA template and to the nascent transcript presenting a possible obstacle for other RNA polymerases [6]. Wind and colleagues have shown in biochemical studies that TFIIS promote arrested RNA polymerase to cleave the displaced 3'-end of the nascent transcript, generating a new 3'-end that is correctly aligned with the DNA template in the active site, and now the elongation reaction can continue from the newly generated 3' –end [1,4]. The displaced nascent transcript can be truncated up to 14 nucleotides [7]. Interestingly, the cleavage activity is stimulated by transcription factor TFIIS in eukaryotes and GreB in prokaryotes, but the reaction is performed by the polymerase itself [8-10].

In an *in vivo* experiment, TFIIS has been shown to associate with an elongating RNA II polymerase when *Saccharomyces cerevisiae* was grown under stress conditions [11]. Reactive oxygen species such as hydrogen peroxide can cause oxidative stress and induce lesions on DNA that block RNA II polymerase from transcribing. Unpublished work from the Kane laboratory has shown that *Saccharomyces cerevisiae* lacking TFIIS exhibits a higher percent survival rate than yeast containing TFIIS in the presence of hydrogen peroxide suggesting that TFIIS plays an important role in regulating gene expression in yeast under oxidative stress [12]. The objective of this project was to identify genes regulated by TFIIS *in vivo* via a global genomic approach using DNA microarrays. The arrays used cDNAs prepared from mRNAs obtained from hydrogen peroxide treated cells containing the TFIIS gene and ones lacking TFIIS.

Materials and Methods

Growth and Peroxide Treatment of CH1305 (wt) and CMKY4 (mut)

Two yeast strains were used in this experiment: CH1305 containing wild type TFIIS and CMKY4 lacking TFIIS. Overnight cultures were set up by inoculating colony in 5 ml of synthetic complete (SC) media and grown at 30°C. The overnight cultures were diluted to a total volume of 200 ml with an optical density (OD_{600}) of 0.025 and grown for 12 h at 30°C to reach mid-log phase (OD_{600}= 0.03-0.05). Cell suspensions were pelleted at 3500 RPM for 5 minutes in a table top clinical centrifuge. The cells were resuspended in 100 ml ddH_2O and pelleted again at 3500 RPM for 5 minutes. The cells were then resuspended in 200 ml 1X PBS to yield an OD_{600} of 0.5. Aliquots of peroxide untreated cells were taken out and diluted to 1:10,000 before plating on SC agar plates grown in 30°C incubator for 3 days. The remaining cell suspensions were treated with 30 mM hydrogen peroxide for 30 minutes at 30°C with shaking at 190 RPM. Immediately after treatment, cell suspensions were diluted with 400 ml cold, sterile ddH2O. Aliquots of peroxide treated cells were taken out and diluted to 1:10 and 1:100 before plating on SC agar plates grown in 30°C incubator for 3 days. The remaining cell suspensions were transfer to a 1 liter centrifuge bottle and pelleted at 4500 RPM for 20 minutes. Pellets were either stored at -80°C or taken to the next step of the experiment: RNA extraction.

RNA preparation

Cell pellets were resuspended in 12 ml of AE buffer (50 mM NaOAC, 10 mM EDTA, pH 5.2) and transferred to an Oak Ridge tube (RNase-free). To each sample, 960 uL of 20% SDS was added and the mixtures were vortexed. Twelve mL of saturated phenol (unbuffered, pH ~4.3) were added. The samples were then incubated at 65°C for 10 minutes with vortexing every minute. The suspensions were spun at 10,000 RPM for 15 minutes. Total RNA from the supernatant was extracted with phenol/ chloroform and precipitated with isopropanol overnight at -20°C. The RNA was pelleted by spinning in an SS-34 rotor at 12,000 RPM for 40 minutes. The pellet was washed with 70% ethanol, air dried, and resuspended in DEPC H_2O. Concentration of total RNA was determined by optical density measurement. The quality of the RNA was verified by running an aliquot out on an agarose gel. Oligo-dT Spin Column (Amersham) was used to purified mRNA from the total RNA. The amount of total RNA from each sample used per column was 1.25 mg producing approximately 25 ug of mRNA.

Microarrays

The purified mRNAs from the peroxide treated TFIIS mutant and wild type yeast strains were used to generate cDNA by reverse transcription. The RNA in the reverse transcription reaction was degraded by addition of NaOH. The reaction was cleaned up using a Microcon 30 filter. The cDNA from the wild type cells were labeled with Cy3 dye and the mutant with Cy5 dye. The labeling reaction was quenched with hydroxylamine and the reaction was cleaned up with a Zymo column. The Cy3 and Cy5 labeled cDNAs were hybridized onto slides with the yeast genome. The slides were scanned using GenePix 400B Microarray Scanner. The data was analyzed using Genepix software to compare the expression level of genes involved in oxidative stress regulated by TFIIS.

Results and Discussions

Obtain Successfully the Peroxide Phenotype

The peroxide phenotype is when a yeast strain lacking TFIIS exhibits higher percent survival than a strain containing wild type TFIIS in the presence of peroxide as illustrated in figure 1. The mutant treated sample has approximately four times more colonies than the wild type treated sample (figure 1). This observation suggests that TFIIS regulates the expression of certain genes during oxidative stress.

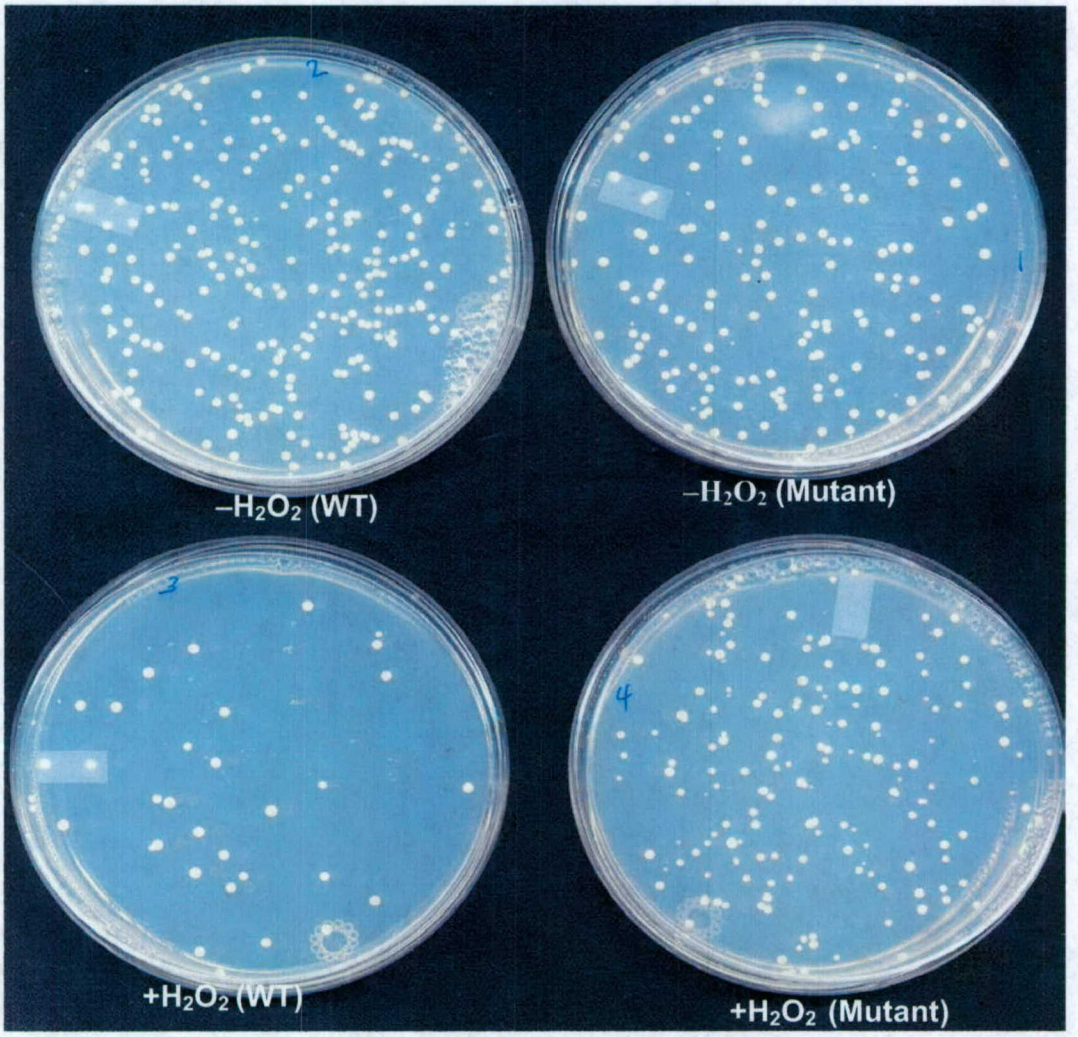

Figure 1. Peroxide phenotype. Top two plates are the wild type and mutant TFIIS yeast samples that were not treated with peroxide and both displayed similar growth. However, the bottom left plate of treated TFIIS wildtype strain exhibits a drastic decrease in survival compared to the treated strain lacking TFIIS.

Isolate Intact Total RNA

Working with RNA is very difficult compared to DNA because of the ubiquitous presence of RNase on nearly every surface. Since this experiment requires the isolation of total RNA from peroxide treated cells, great care was employed to prevent the RNA samples from being contaminated with RNase. To confirm the integrity of the RNA samples, aliquots of the samples were ran out on an agarose gel. As shown in figure 2, the expected band patterns of the ribosomal RNAs are seen suggesting that the RNA samples have not been degraded by RNase.

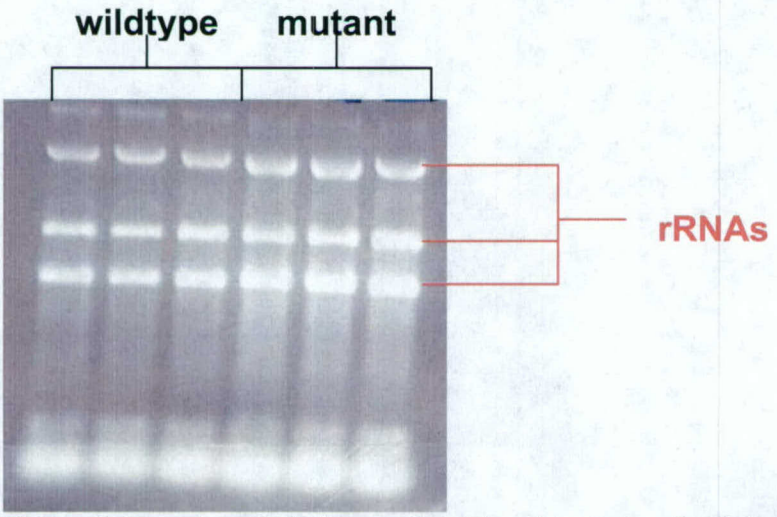

Figure 2. Integrity of RNA samples. The discrete bands of the ribosomal RNAs are seen in all samples indicating that the RNA has not been degraded.

Oxidative stress occurs when the concentration of oxidants exceed that of the antioxidant buffering capacity of the cell. To protect its cellular constitutents from reactive oxygen species (ROS) such as peroxide, the budding yeast *Saccharomyces cerevisiae* employs defense mechanism that consists of small molecules that act as general radical scavengers to reduce ROS and several enzymes that can remove oxygen radials or repair the damage caused by oxidative stress. Perhaps TFIIS plays a role in regulating the defense mechanism against oxidative stress by regulating the transcription of some of the genes involved in the defense mechanism. Preliminary results show that in TFIIS deleted cells relative to wild type TFIIS cells, seven proteins involved in metabolic pathways are upregulated at least two fold and two proteins involved in oxidative stress response are downregulated at least two fold when these types of cells are treated with peroxide (table 1). Our findings suggest that these proteins might be regulated by TFIIS in *Saccharomyces cerevisiae* during oxidative stress.

Name	Ratio of Medians mutant /wt	"MOLECULAR FUNCTION"'
TDH2	0.267	'GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE'
RHR2	0.285	'DL-GLYCEROL-3-PHOSPHATASE'
PGI1	0.427	'PHOSPHOGLUCOISOMERASE'
EGD2	0.44	'GAL4 ENHANCER PROTEIN
PGK1	0.465	'3-PHOSPHOGLYCERATE KINASE'
TKL1	0.484	'TRANSKETOLASE 1'
PDA1	0.507	'ALPHA SUBUNIT OF PYRUVATE DEHYDROGENASE (E1 ALPHA)'
TRR1	1.969	'THIOREDOXIN REDUCTASE'
RPL18A	0.338	'PROTEIN COMPONENT OF THE LARGE (60S) RIBOSOMAL SUBUNIT
RPL8A	0.353	'RIBOSOMAL PROTEIN L4 OF THE LARGE (60S) RIBOSOMAL SUBUNIT
RPL2B	0.369	'PROTEIN COMPONENT OF THE LARGE (60S) RIBOSOMAL SUBUNIT
RPL8B	0.37	'RIBOSOMAL PROTEIN L4 OF THE LARGE (60S) RIBOSOMAL SUBUNIT
RPL23B	0.371	'PROTEIN COMPONENT OF THE LARGE (60S) RIBOSOMAL SUBUNIT
RPS0A	0.372	'PROTEIN COMPONENT OF THE SMALL (40S) RIBOSOMAL SUBUNIT

Table 1. Microarray Data. Proteins involved in metabolic pathways, oxidative stress, and ribosomal proteins appear to be differentially expressed between the wild type and mutant TFIIS yeast strains in the presence of peroxide. **Key: blue, metabolic pathways; green, oxidative stress; red, ribosomal protein.**

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Project Title: Molecular Determinants of Epimorphin Branching Morphogenesis

Abstract:

Identification of the signals controlling the development of the mammary gland epithelial ductal system has been an intensive topic of investigation. This process can be modeled in 3-dimensional culture systems in which mammary epithelial cells are grown in collagen-I gels. Previous investigations have identified epimorphin (EPM) as an essential morphogen for mammary gland development through branching and luminal morphogenesis. EPM is the extracellular form of the intracellular protein syntaxin-2, a member of the syntaxin family of vesicle fusion mediators. EPM is produced only in the stromal compartment of the mammary gland, but binds to nearly all cultured luminal epithelial cells, suggesting the presence of a cell surface receptor on luminal epithelial cells. In this study, we identify the domain of EPM responsible for directing branching activity in the 3D collagen assay. Using the structure of the highly homologous syntaxin-1A (which shows no activity as a mammary epithelial morphogen) as a basis for a homology model of EPM, we selected a region of EPM that we predict to be the active domain, identified four amino acids that are distinct between EPM and syntaxin-1A, and performed site-specific mutagenesis of syntaxin-1A to change all four to those that are found in EPM. We found that this hybrid product (which we have named HS 1→2) reproduced the activity of EPM in the 3D collagen assay. By creating an active morphogen from an inactive template, we have identified the active domain of EPM.

Introduction:

Branching morphogenesis is an essential process for development of mature, functional mammary glands. During puberty, mammary gland end buds proliferate and invade the mesenchymal fat pad, establishing a network of ductal branches. During pregnancy, the epithelium continues to grow, developing additional lobuloalveolar structures (Radisky et al., 2003). *In vivo*, these developmental processes are dependent on communication between the stroma and the epithelium; these interactions direct branching morphogenesis, for tubule initiation and extension, as well as luminal morphogenesis, for formation of secretory acini. Branching morphogenesis can further be divided into two distinct processes (Wiseman and Werb, 2002), as primary branches form from separation of the advancing terminal end buds, while secondary and tertiary branches emerge from mature ducts. Both types of branching morphogenesis is dependent on an epithelial-to-mesenchymal transition, because as the epithelium branches, the stroma must undergo remodeling to make room for and provide support to the developing epithelial tubes (Metzger et al., 1999).

While several types of growth factors can stimulate mammary epithelial cell proliferation, epimorphin is a required morphogen to provide direction and orientation to the developing structures (Hirai et al., 1998). Previous studies have shown that matrix metalloproteinases (MMPs) are needed for mammary branching morphogenesis induced by growth factors and epimorphin (Simian et al., 2001). Epimorphin-treated mammary epithelial cells were found to increase levels of MMP-2, MMP-3, and MMP-9 (Simian et al., 2001) and inhibition of the MMPs completely blocked the branching activity (Hirai et al., 2001). These studies show epimorphin branching morphogenesis is mediated through MMPs and the pathway connecting epimorphin and implicate this effector is an important target for investigation. In the mammary gland, epimorphin is present at the surface of both stromal fibroblasts and myoepithelial cells and plays a large role in mammary gland morphogenesis. In vitro experiments have shown that epimorphin directs distinct

morphogenic pathways in mammary epithelial cells cultured in 3D collagen. Depending orientation of presentation, epimorphin stimulates strikingly different morphogenic processes. Polar presentation of epimorphin to the outer surface of cell clusters lead to branching morphogenesis. However, the presentation of epimorphin around every cell (apolar) in the cluster results in luminal morphogenesis where a large central lumen forms in cystic structures (Hirai et al., 1998).

Although epimorphin was first identified as a morphogen through the blocking of its activity using an epimorphin monoclonal antibody (Hirai et al., 1992; Gumbiner, 1992), the same molecule was later identified as the extracellular form of syntaxin-2, a functionally distinct molecule and a member of the syntaxin family of proteins that function in vesicle fusion (Bennett et al., 1993; Pelham, 1993). Although epimorphin has yet to be crystallized, syntaxin structure has been extensively studied through nuclear magnetic resonance (NMR) and crystallography. Different members of the syntaxin family are highly homologous, and are likely to be structurally similar. The common characteristic that defines the syntaxin family of proteins is a conserved C terminal region that generally precedes a transmembrane domain. This C-terminal region also known as the SNARE domain is believed to be directly involved in membrane fusion. Through NMR spectroscopy studies, residues 27-146 of syntaxins consist of an independently folded domain with a three-helix bundle structure. The interface between the second and third helices forms a long groove that exhibits substantial residue conservation between different syntaxins. This groove is an ideal binding site for a helix of another protein that can pack against it completing the four-helix bundle. For syntaxin, Synaptobrevin/VAMP and SNAP-25, form a tight complex with the SNARE domain that is known as the core complex or the SNARE complex. Formation of this complex is believed to provide the driving force for fusion of vesicles with the plasma membranes (Fernadez et al., 1998).

Deletion analyses have shown that the functional domains of epimorphin and syntaxin can be distinguished (Hirai et al., 1998, Weimbs et al., 1997). Mammary luminal epithelial cells respond to epimorphin but do not express it, and are surrounded by epimorphin expressing mesenchymal cell populations, demonstrating the existence of epimorphin receptors on the outside of the luminal epithelial cells, the properties of which can be investigated using cell adhesion assays. Studies have shown that the cells attached to the three alpha helical region (H12) of recombinant epimorphin but not to the region containing the SNARE helix and transmembrane domain (H3). Domain minimization of epimorphin activity was also tested using branching assays, where SCp2 (mammary epithelial cells) were embedded in collagen-1 in the presence or absence of recombinant epimorphin. The H12 region was shown to be sufficient to induce branching morphogenesis while the H3 was not (Hirai et al., 1998). Thus, the SNARE domain is dispensible for epimorphin cell binding and morphogenesis activity; in contrast, deletion analyses of syntaxin proteins have suggested that the SNARE domain is sufficient by itself to mediate vesicle fusion (Weimbs et al., 1997)

While the three helix domain of epimorphin is active by itself to mediate cell binding and morphogenesis, numerous biochemical and structurual studies have shown that this structure prefers to bind to a fourth alpha helix to form a four-helix bundle. These observations are suggestive of a hypothetical fourth helix, perhaps on the epimorphin receptor, that may bind to epimorphin in the cleft between the second and

third helix. If so, then this cleft is predicted to be the critical morphogenic determinant of epimoirphin; it is the objective of this study to evaluate this prediction.

Materials and Methods:

Cells

The functionally normal and nontumorigenic mouse mammary epithelial cell line, SCp2, was maintained in growth medium (DME/F12 supplemented with 2% FBS, 5ug/ml, insulin and 50 ug/ml gentamicin).

Generation of Recombinant Epimorphin

Expression constructs were generated by PCR amplification using cDNA for mouse EPM or human syntaxin-1 as template and were cloned into a pET27 expression vector, and subsequent site-directed mutageneses was performed using the Stratagene Quickchange PCR-based mutagenesis kit. Recombinant epimorphin (HS EPM) is identical to endogenous epimorphin except that it lacks the N-terminal 26 amino acids, as well as the linker, SNARE helix, and transmembrane domains. Recombinant syntaxin-1a (HS 1A) is derived from the three-helix bundle domain of Syntaxin 1a (homologous to HS EPM). Recombinant HS1→2 is derived from HS 1A with the proposed active site residues mutated to the ones in Syntaxin-2. The proteins were tagged with six histidine residues and expressed in *Escherichia coli*. The *E. coli* was lysed with BugBuster reagent supplemented with protease inhibitors and benzonase and purified over Ni columns. For use in cell culture, recombinant proteins were dialyzed against 1X PBS and filtered under sterile conditions. Proteins were diluted to a concentration of 0.76 mg/ml using 1X PBS.

3D Cell culture

The cell clusters were prepared as follows: agarose was heated in DME/F12 (final 2%) and 1 ml of the solution was added to each well of 24-well plates. After the agarose gelled, 1.5ml of growth medium was added to each well and incubated for 1 hr at 37° C in a CO_2 incubator. This medium was then discarded and SCp2 cells suspended in 500ul of growth medium containing 1,000U of desoxyribonuclease I (DNase I) were added on top of the agarose gel and incubated at 37° C with gentle rotation (100rpm) for 24 hr, which yielded rounded and well packed cell clusters. Unclustered single cells were removed by centrifugation and the clusters were then washed three times with DME/F12. Cell clusters were embedded in type I collagen gels. Acid-soluble collagen (Cellagen) was mixed gently on ice (8 vol) with 1 vol of 10X DME/F12 and 1 vol of 0.1N NaOH. 100 µl of the collagen solution was added into each well of a 48 well plate, which was then incubated at 37° C to allow for polymerization of the basal collagen layer. The cell clusters were suspended in growth medium and 10 µl of the suspension (24-40 clusters) was mixed with 10 µl of laminin, 10 µl of protein solution and 70 µl of the collagen solution, and poured onto the basal collagen layer and placed at 37° C for gelation. After gelation of the collagen, 200 µl of growth medium containing 10% protein solution and 50 ng/ml EGF (epidermal growth factor) was added on top. Cultures were maintained at 37° C in tissue culture incubators and liquid media was changed every other day.

Results:

Development of homology model

Key residues in the predicted functional domain were identified by homology modeling and visualization using the DeepView Swiss-Pdb Viewer. A model structure of epimorphin was created by threading the sequence of epimorphin into the published crystal structure of syntaxin-1a. In the closed conformation structure of syntaxin-1a, the SNARE helix is binding as a fourth helix in the cleft between the 2nd and 3rd alpha-helices; accordingly, the SNARE helix was used as a model for a hypothetical helix that binds to the three alpha helical region of epimorphin. Together, these were used as a model to identify the amino acid residues of the epimorphin three-helix bundle with side chains pointing towards the proposed hypothetical fourth helix.

Morphogenesis

Of the residues in the homology model of epimorphin that were predicted to be in contact with the hypothetical fourth helix, four were found to be different between syntaxin-1a and epimorphin. To create the HS1→2 recombinant protein, these four residues were changed on the syntaxin-1a template to those found in syntaxin-2 (epimorphin), using site-specific mutagenesis with the Stratagene Quik Change Kit. In helix 2, argininine90 was mutated to a methionine and asparate102 was mutated to a glutamate; in helix 3, phenyalanine142 was mutated to a tyrosine and serine146 was mutated to a cysteine.

Branching activity and Cell Adhesion

If the proposed active site of the protein carried key residues required for epimorphin activity, it was expected that HS EPM would be active in the branching assay, HS Syn1a would be inactive, and HS1→2 would regain branching activity. Using these recombinant proteins in branching assays, SCp2 cells were embedded in collagen I and incubated with 5% serum + GI plus EGF and 10% volume of recombinant protein solution. Untreated cells, or cells treated either with growth factors or EPM alone (but not both) showed no branching morphogenesis. However, cells simultaneously treated with growth factors and epimorphin did exhibit branching activity, depending upon the form of epimorphin that was used. After 10d, the cells incubated with HS EPM and EGF exhibited abundant branching, while the corresponding HS Syn1a-treated cultures showed only small stubby branches. The recombinant protein HS1→2 was found to be active, beginning to stimulate branching after 4 d, demonstrating that our mutations had coferred morphogenic activity upon an inactive template.

Discussion:

Structural insights can be used in functional studies of homologous proteins

In this study, we used information from the crystal structure of the homologous protein syntaxin-1a to develop a hypothesis about the function of epimorphin, and to target amino acids predicted to be involved in the epimorphin morphogenesis domain. Use of this strategy relied upon the fact that epimorphin and syntaxin have very distinct functions and functional domains, but are encoded by the same gene. These distinct functions are reflective of the different localizations of the epimorphin/syntaxin-2 protein: epimorphin is localized to the extracellular surface of the plasma membrane, while syntaxin is localized to the cytoplasmic face. Epimorphin is also unusual in that the protein has no canonical exocytosis-targeting signal sequence, and as such, appears to

cross membrane without transiting through the endoplasmic reticulum and golgi apparatus. Other examples of proteins that exit cells without using the classical protein secretion route include fibroblast growth factors 1 and 2 and interleukin-1B (Siders et al., 1995), which also lack signal sequences but which have well-characterized functions outside of the cell. Epimorphin/syntaxin-2 can be contrasted from these proteins in that it appears to have completely distinct functions depending upon its membrane orientation. Intracelullarly, syntaxin-2 mediates membrane fusion, while extracellularly, epimorphin acts as a morphogen. Epimorphin/syntaxin-2 is not unique in its topologically distinct functionalities, as it is one of a subcategory of proteins that lack signal sequences but also have distinct extracellular and intracellular functions. Other examples of these proteins include phosphohexose isomerase, autocrine motility factor, RHAMM/CD168, Galectin-1, HMGB1/amphoterin, tissue transglutaminase, and theioredoxin/ADF (Radisky et al., 2003). Many of these proteins have been studied structurally in one topological/functional context, so our strategy to identify functional domains of epimorphin through structural analysis of the homologous syntaxin-1a could also be applied to other members of the family of proteins with distinct extracellular and intracellular functions.

The discovery of the active site of epimorphin may also have applicability towards investigations that provide insight into mechanisms involved in the development of breast cancer. The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) regulates the expression of many genes involved in proliferation and terminal differentiation. C/EPBβ has been shown to be important for normal mammary gland morphogenesis and epithelial cell fate determination (Robinson et al., 1998). C/EBPβ functions as a homo- or heteromeric dimer of its two constituent isoforms: LAP (liver activating protein) and LIP (liver inhibiting protein) (Descombes and Schibler, 1991; Buck et al., 1994). LAP and LIP are derived from alternative translation initiation from the a single mRNA transcript with LAP being the full-length form containing a transactivating, dimerization, and DNA-binding domains, while LIP is a truncated form lacking the transactivating domain. LAP and LIP are mutually antagonistic, and alterations in the relative ratio of expression of the LAP and LIP can lead to dramatic alterations in expression levels of of genes signaling either differentiation or proliferation. The two isoforms are crucial for mammary gland development and are also known to be overexpressed in breast cancer. Investigations of WAP-LIP mice, which express increased LIP selectively in mammary epithelial cells, show that LIP can induce epithelial proliferation and the formation of mammary hyperplasias and that a LIP-initiated growth cascade may be susceptible to additional oncogenic hits, which could lead to the initiation and progression of neoplasia (Zahnow et al., 2001). Intriguingly, epimorphin is the only known regulator of C/EBPβ (Hirai et al., 2001), and it has been found to increase the overall levels of C/EPBβ and to increase the ratio of LIP to LAP in both culture and in the WAP-EPM transgenic mouse (Hirai et al., 2001), which also shows significantly elevated rates of malignancy (J.L.Bascom *et al.*, manuscript in preparation). The investigation of the mechanism of how epimorphin controls the levels of the protein isoforms necessitates the identification of the epimorphin receptor which has yet to be found. The results described here detail the identification of the epimorphin active site, a key step in the path towards discovery of the receptor. Additionally, the identification of the binding cleft may be useful for creating an α-helix designed to bind

to the active site tight enough to prevent the binding of epimorphin to its receptor and therefore blocking the effects that epimorphin binding. Given that epimorphin acts to increase the ratio of LIP to LAP, which signals for increased malignancy, inhibiting epimorphin binding could have potential as a cancer preventative or therapeutic agent.

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Project Title: Control of Chromosomal DNA Structure by Microenvironmental Cues

Introduction

Generally, the DNA sequence encoding a gene has to be exposed in order for it to be transcribed and expressed. Puck et al. (1990) have shown that the DNA from cells in a reverse-transformed state is 3 times more sensitive to DNase I than the DNA from transformed cells. This led him to hypothesize that changing higher order chromatin structure was associated with changes in sequestration and exposure to DNase binding sites.

ALU sequences have been shown to be present repeatedly and associated with gene expression in cancer. Induction of heat shock protein expression leads to the production of mRNA containing ALU RNA and this induction is correlated with an increase in the accessibility of ALU chromatin to restriction enzyme cleavage (Kim et al., 2001). Furthermore, most ALU elements contain functional RNA polymerase III promoters and inhibit transcriptional interference (Willoughby et al., 2000).

In a recent study, Maniotis et al (manuscript in preparation) showed that the ALU I DNA sequences of normal stromal cells are less resistant to restriction cleavage than ALU I sequences in malignant tumor cells. Based on these observations, these authors hypothesized that the genome structure changes as a tumor cell becomes more aggressive.

On Matrigel highly invasive cells form looping patterns, while forming only monolayers on 2D. Normal breast cells form polar, acini-type structures in Matrigel, but do not form these structures on Type 1 collagen. Maniotis observed a difference in digestion sensitivity of chromatin by ALU that ECM molecules confer on normal fibroblasts, MCF10a breast epithelial cells, MDA-MB231 breast cancer cells, and melanoma cells of low and high invasive potential. They found that by manipulating microtubular assembly, intermediate filaments, and actin they could alter chromatin sensitivity to ALU I, suggesting that ECM acts through the cytoskeleton to vary the degree of exposure of ALU I binding sites.

Materials and Methods

- 0.1 mg/mL DNAase I
- 100 ug/mL Ethidium Bromide
- non-malignant cell lines: S1 early and late passages, MCF 10A, P2
- malignant cell lines: T4, MDA-MB231, MCF 7

The P2 cells were plated onto 4-well chamber slides with a cell density of $1x10^6$ cells per well and with supplemented DMEM F-12 media (500 uL gentamicin, 500 uL of 5 mg/mL insulin, and 10 mL of 10% fetal bovine serum) and allowed to grow to be about 70-80% confluent. The MCF 10A and S1 cells were grown in supplemented DMEM/F-12 media (5 uL of 20ug/mL EGF, 0.2uL of 5 mg/mL cholera toxin, 20 uL of 5 mg/mL insulin, 1 uL of 5 mg/mL hydrocortisone, and 500 uL of 100% horse serum). The T4 cells are grown in the same media, excluding the EGF. MDA-MB231 cells were grown in Leibovitz L-15 media with 0.5 mL of 10% fetal bovine serum. The S1, T4, and P2 cell lines were also treated with Matrigel drip and on top (extracellular matrix or EHS), as well as collagen drip, for either a 1- or 2-day period. Before beginning the DNAase treatment on the cells, the media was aspirated out of the wells or drained out in the case where the cells were treated with EHS and collagen, and allowed to dry for at least an hour. Non-treated cells were allowed to air dry, while cells treated with EHS/collagen were baked in a 37°C incubator for approximately 1-2 hours. Drying the cells disrupted the cell's membrane, allowing DNAase I access to the chromatin. 0.5 uL of 0.1 mg/mL DNAase I was added to the dried cells and gently spread over a small area. The cells were treated with the restriction enzyme for a 2-hour period. 2 uL of 100ug/mL $EtBr_2$ was then added on top of each treated area. Ethidium bromide attaches to DNA and fluoresces under the microscope, and therefore, was used to determine the extent of digestion by DNAase I. The cells were immediately placed on ice after the 2-hour treatment period in order to halt DNAase I activity. Cells were then viewed under the Nikon microscope.

Results

There is evidence that the malignancy of a cell affects the extent of its DNA digestion. Cell digestion was determined by the degree of ethidium bromide fluorescence, since ethidium bromide binds to DNA and is therefore a marker for the amount of DNA present in the cell. The amount of digestion was compared among cell lines at the zero and two hour period. The extent of DNA digestion in non-malignant cells, such as the MCF10A, S1 early passage cells, and P2, was much greater than that of malignant cells at the 2-hour mark, such as the MDA-MB231, MCF7, and T4 cells, suggesting that the chromatin in malignant cells are more sequestered, and therefore, more resistant to digestion. We used S1 early and late passages and T4 cells because, since they are all part of the same cell line, we could see a progression of digestion as the cell line became more malignant. The non-malignant S1 early passage cells showed a significant amount of digestion over the 2-hour treatment, but the amount of digestion decreased as the cells became more malignant in the S1 late passage, which more closely resembled the amount of digestion occurring in the malignant T4 cells.

The affect of the extracellular matrix on the sequestration and exposure of chromatin was also studied, showing that the presence of ECM decreased the extent of digestion in cells. Various amounts of EHS were used, varying from 1 to 6% EHS, in order to determine whether the extent of chromatin digestion by DNAase I was influenced by the thickness of Matrigel. The results show greater resistance to digestion as the thickness of Matrigel increased.

Future

Further DNase I treatments need to be repeated on the S1 and T4 cell lines in order to obtain conclusive evidence. In addition, further experiments need to be done in

order to determine the effect of the extracellular components, such as laminin and collagen, on the resistance of S1 early and late passages and T4 cells when they are treated with DNase I. Finally, all the cell lines used in this experiment need to be treated with the ALU1 restriction enzyme, which was the enzyme in the original Maniotis experiment, instead of DNase I in order to determine whether our results corroborate with Maniotis' results.

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Project Title: Mechanochemical Signaling for β-Casein Expression

Previous research in Mina Bissell's laboratory has found that both physical and chemical signals are required for induction of transcription of the milk protein β-casein in mouse mammary epithelial cells; that in addition to chemical signals from the extracellular matrix and hormones, the cells also required physical alterations of cell shape in order to functionally differentiate. Here, we have explored the relationship between chemical and physical inputs of cell function by subjecting EpH4 mouse mammary epithelial cells cultured in drip conditions to different levels of strain, and monitoring the induced changes in β-casein transcription and translation by RT-PCR and western blot. As a preliminary study, we measured the rate of induction of β-casein in cells cultured on the flexible substrata in differentiation medium (pl) or in differentiation medium to which EHS has been added. We found that the cells rapidly expressed β-casein when exposed to EHS, and that sustained incubation led to induction of β-casein even in cultures to which EHS had not been added (Figure 1). Future studies will involve dynamic and static stretching experiments.

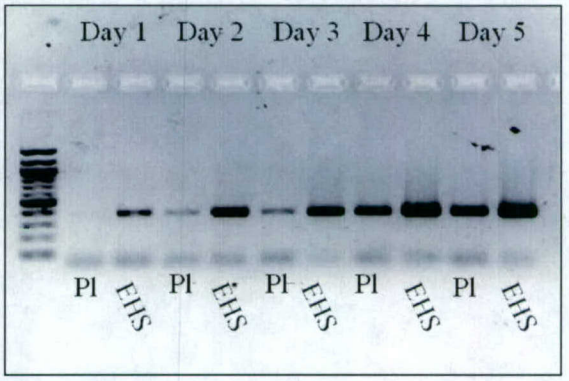

Figure 1. Expression of β-casein on a flexible substratum.

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Project Title: The Differential Expression of Double-stranded RNA-dependent Protein Kinase (PKR) and p53 Apoptosis Effector Related to PMP-22 (PERP) in Estrogen Protected vs. Unprotected Lewis Rats
Abstract

It has been known that early pregnancy confers protection against breast cancer in women. The same phenomenon has been observed in rats and mice. Experimental evidence has shown that short-term hormone treatment with pregnancy level estrogen is highly effective in decreasing the incidence of mammary cancers. To understand this protective mechanism, microarray analysis was performed and it was found that specific genes were differentially expressed in the hormone protected and unprotected rats. Among the genes that were persistently upregulated in the hormone-protected rats were Double-stranded RNA-dependent Protein Kinase (PKR) and p53 Apoptosis Effector Related to PMP- 22 (PERP). Both genes have been found to be involved in regulating apoptosis, which has been hypothesized to be altered in the protective group. The purpose of this study was to validate the findings of the microarray analysis through Real-Time RT-PCR (Reverse Transcriptase PCR), focusing on the preferential gene expression of PKR and PERP. Nine-week old female Lewis rats were treated with 10 μg (unprotected) or 200 μg (protected) of estradiol in silastic capsules for 3 weeks. Control rats received silastic capsules with no hormone. The rats were terminated 8 weeks after the removal of the hormone treatment. Mammary RNA was used for RT-PCR analysis, where the gene expression levels of PKR and PERP were compared between the different groups. Data analysis showed a 4.9-fold increase in expression of PERP and an 8.6-fold increase in expression of PKR in the protected group.

Introduction

It has been a known fact that early pregnancy confers protection against breast cancer in women. This is the only known physiological mechanism that can drastically reduce the risk of mammary carcinogenesis in women of all ethnic backgrounds. Women who have undergone a full term pregnancy before the age of 20 have a 50% reduced risk of breast cancer, compared to nulliparous women (1-4). The same phenomenon of parity protection against chemically-induced mammary carcinogenesis is also observed in rats (5-11) and mice (12-14) that undergo a full term pregnancy compared to nulliparous animals.

The mechanism for this protective effect has not been fully elucidated, although it has been thought to be related to the increases in hormone levels during pregnancy that cause persistent changes in the systemic hormonal environment in parous females. Experimental evidence has shown that short-term hormone treatment with pregnancy level estrogen, or estrogen and progesterone is highly effective in decreasing the risk for mammary carcinogenesis (15-19).

To understand this protective mechanism, microarray analysis was performed on RNA extracted from mammary tissues of hormone treated rats and it was found that specific genes were differentially expressed in the hormone-protected and unprotected groups (20). Growth-promoting genes such as Cyclin-Dependent Kinase2 and 4 (CDK2, 4) are down-regulated in the protected group while Growth-inhibiting genes, such as PKR and PERP are upregulated.

PKR (double-stranded RNA-dependent protein kinase) was first identified as a mediator of the antiproliferative and antiviral actions of interferon. The expression of PKR and its action is induced by the overexpression of the transcription factor, E2F-1 in response to hyperproliferation signals such as tumor necrosis factor α (TNF-α), and interferon. The deregulation of E2F-1, which induces cell cycle progression at the G_1/ S

checkpoint of the cell cycle leads to the autophosphorylation of PKR, initiating a signal transduction cascade which can eventually lead to apoptotic cell death. PKR has also been found to regulate NF-κB, and p53, which are crucial in regulation of cell proliferation as well as induction of apoptosis in a variety of tumor cell lines (21, 22).

Similarly, PERP (**p**53 apoptosis **e**ffector **r**elated to **P**MP-22) is also a pro-apoptotic gene. As its name implies, PERP is a member of the PMP-22/*gas*3 family of tetraspan transmembrane proteins involved in the regulation of cell growth. PERP has been found to be involved primarily in the p53-dependent apoptotic pathway, and like PKR, is also induced in response to E2F-1-driven hyperproliferation. It has been found that PERP is expressed at higher levels in apoptotic cells compared to G_1- arrested cells. This suggests that PERP, whose promoter already contains two p53 binding sites, is not only tightly associated with p53 but may also have additional regulatory elements in its promoter that preferentially upregulates PERP in apoptotic cells. PERP's sequence similarity to the calcium channel γ-subunit suggests pore or channel activity, which probably allows these extra apoptotic factors to pass through the membrane and work to promote apoptosis (23, 24).

It has been suggested that short-term high pregnancy level estrogen treatment has a persistent effect in upregulating genes that inhibit cellular proliferation. Since it has been established that PKR and PERP function to inhibit cell proliferation and thus, induce apoptotic cell death, it would be plausible that both these genes will be upregulated in the protected group.

Recognizing the persistent expression profiles of these genes can be used to further understand the molecular mechanism of the protective effect of hormone treatments. Moreover, these differential gene expression patterns can be used as biomarkers for the efficacy of hormone treatments against mammary carcinogenesis. Thus, it is important to confirm the microarray data through RT-PCR.

Materials and Methods

Animals

Seven-week old virgin Lewis rats were purchased from Harlan Sprague-Dawley (Indianapolis and San Diego). The rats were housed in a temperature-controlled room with 12-h light/dark schedule. They were fed food (Teklad 8640; Teklad, Madison, WI) and water ad libitum. All of the procedures followed University of California Animal Care and Use Committee guidelines.

Estradiol Treatment

All doses of Estradiol-17-β (Sigma) were packed in the silastic capsules (size 0.078 inch i.d. x 0.125 inch o.d., 2 cm in length; Dow Corning Corporation, Midland, MI) in a cellulose matrix. Control animals received empty silastic capsules. All silastic capsules were dorsally implanted s.c. All capsules were primed before implantation by soaking in media 199 (GIBCO) overnight at 37 °C.

Persistent effect of different doses of estradiol on gene expression patterns in mammary gland

When the rats were 9 weeks old, they were divided into 3 groups; each group consisted of 3 rats and received one of the following treatments: (i) Control, (ii) 10 μg estradiol (unprotective), and (iii) 200 μg estradiol (protective). Each treatment lasted 3 weeks and at the end of the treatment, the silastic capsules were removed from the

animals. Eight weeks after hormone removal, the rats were terminated. Mammary glands were removed and immediately snap-frozen in liquid nitrogen and stored at -80°C.

RNA Isolation

The mammary tissues that were stored at -80°C were used for RNA isolation. RNA was isolated using Trizol (Invitrogen). Total RNA was then subjected to DNAse treatment (Ambion) and was quantified using a Shimadzu UV-1201 UV-VIS spectrophotometer.

RT-PCR

Real Time RT-PCR analysis was performed using the iCycler iQ Real Time PCR Detection System. Each reaction volume was 25 µl, where each component volume was as follows: 12.5 µl 2x QuantiTect SYBR Green RT-PCR master mix, 1 µl each of 50 pmol/µl forward and reverse primers (see below), 9 µl RNase-free water, 0.25 µl fluorescein, 0.25 µl QuantiTect RT Mix, and 1 µl of 200 ng/µl template RNA. The QuantiTect SYBR Green RT-PCR kit was used according to the manufacturer's specifications. Primers were ordered from Qiagen. For PKR, the forward sequence was *5'-CCT GTC CTC TGG TTC TTT TGC T*-3'and the reverse sequence was *5'-GAT GAT TCA GAA GCG AGT GTG C*-3'. For PERP, the forward sequence was *5'-TAA GCC GCT GTC ACT ACT*-3' and the reverse sequence was *5'-CTT CTT CCC ACA CCC TAC C*-3'. The primers were reconstituted in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.4) and were diluted to 50 pmol/µl.

Data Analysis

Computer quantification of gene expression was done by taking the cycle threshold (Ct) values for each sample and calculating the fold difference according to a specified formula (BioRad and Applied Biosystems SYBR Green Kit).

Results

For both PERP and PKR, there was relatively higher gene expression in the protected group compared to the unprotected group. Analysis of the differential expression of PERP showed no significant increase in the unprotected group and a 4.9-fold increase in the protected group when compared to the control (see Figure 1). Similarly, PKR expression was significantly higher in the protected group than the unprotected group when compared to the control. There was an 8.6-fold increase in expression in the protected group, while only a 1.58-fold increase in PKR expression in the unprotected group (see Figure 2). For both genes, the gene expression for the control group was set to 1 and the relative expression of each gene for the protected and unprotected groups was compared to this value.

Figure 1. Differential Expression of PERP in hormone protected (200 µg) and unprotected (10 µg) groups

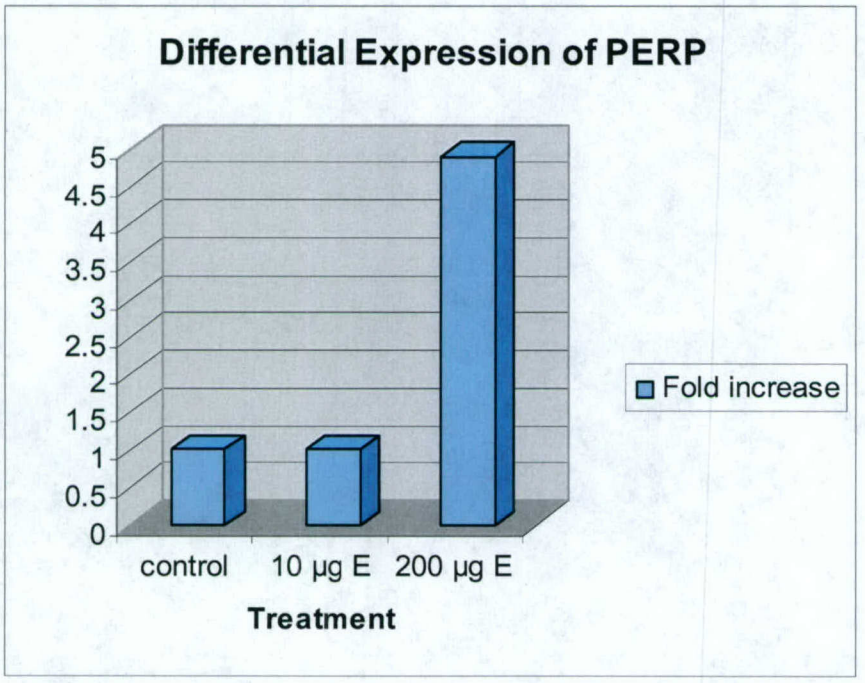
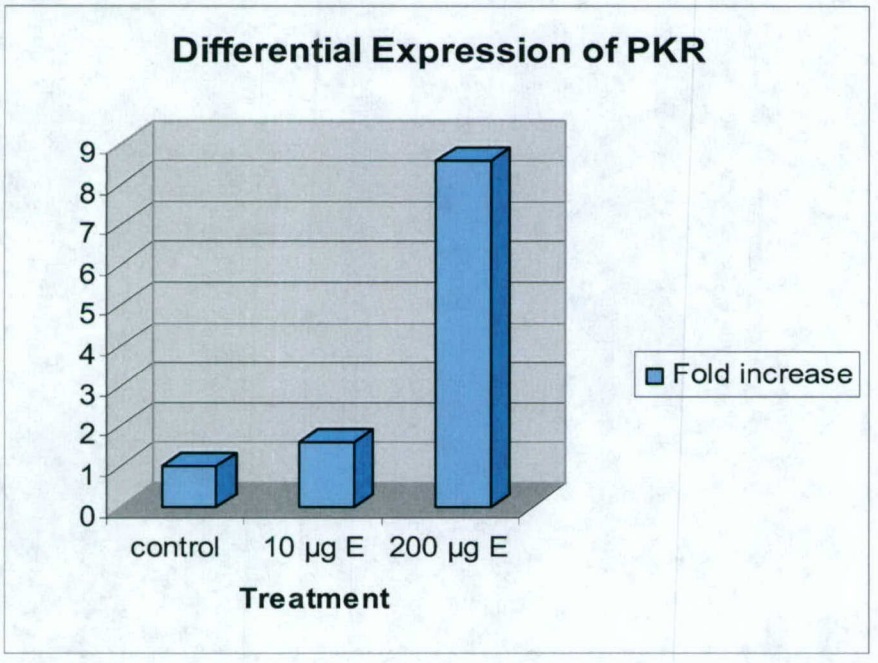


Figure 2. Differential Expression of PKR in hormone protected (200 µg) and unprotected (10 µg) groups



Discussion

It has been shown that pregnancy protects against mammary carcinogenesis in women (1-4). Although this effect is universal, the molecular mechanism for this protection has not been fully understood. It has been thought to be related to the increases in hormone levels during pregnancy that cause persistent changes in the systemic hormonal environment in parous females. Experimental evidence has shown that short-term hormone treatment with pregnancy level estrogen, or estrogen and progesterone is highly effective in decreasing the risk for mammary carcinogenesis (15-19).

The microarray analysis was done to try to understand this mechanism by looking at differential gene expression profiles of a number of genes. Pro-apoptotic genes such as PERP and PKR were among those that were found to be persistently upregulated in the protected group compared to the unprotected group. In this study, the significantly higher gene expression observed in the protected vs. the unprotected groups, confirmed the findings of the microarray analysis.

It has been suggested that short-term high pregnancy level estrogen treatment has a persistent effect in upregulating genes that inhibit cellular proliferation. Since it has been established that both PKR and PERP function to inhibit cell proliferation and thus, induce apoptotic cell death, an increased expression of these genes could counter the increased proliferation and decrease the frequency of transformation that influences the likelihood of mammary carcinogenesis (18). This could be a plausible explanation for the protection conferred by this treatment.

The confirmation of the persistent gene expression profiles of PKR and PERP can be used to further understand the molecular mechanism of the protective effect of pregnancy level hormone treatment, which has been found in previous studies to mimic the protective effect of parity against mammary cancers (15-19). Moreover, these differential gene expression patterns can be used as biomarkers for the efficacy of hormone treatments against mammary carcinogenesis.

Conclusion

Based from this study, the findings of the microarray analysis were confirmed. The pro-apoptotic genes PKR and PERP were persistently upregulated in the protected group compared to the unprotected group. These findings may suggest that an increase in cell death may be one of the mechanisms for the protective effect that is conferred by early pregnancy, as confirmed by the administration of short-term pregnancy level hormone treatments, to mimic this effect. Moreover, these differential gene expression patterns can be used as biomarkers for the efficacy of hormone treatments against mammary carcinogenesis.

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Mina Bissell Lab
Supervisor: Virginia Novaro
Project Title: Estrogen Receptor alpha (ERα) and its regulation by the extracellular matrix (ECM)

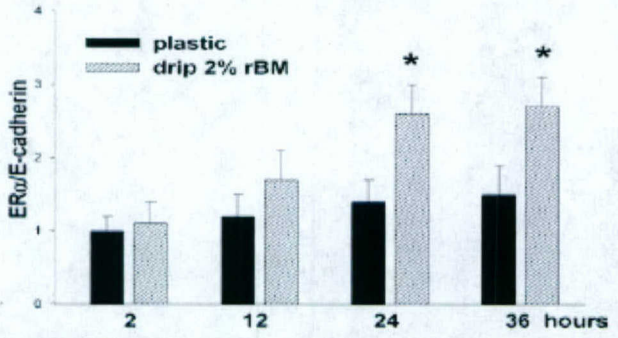
Abstract

Estrogen receptor alpha (ERα) is an important molecule involved in proliferation and differentiation of the cell. Thus a proposed model of interest is the study of normal and malignant cell lines, such as SCp2 and SCg6 respectively, to learn about the conversion from "normal" to malignant cells. It has been shown that the extracellular matrix (ECM) plays a role in the regulation of the ERα levels, specifically collagen-IV and laminin-1 components of the ECM, in primary mammary epithelial cells as well as in SCp2 cells, but not in SCg6 cells. We are now studying the difference in these two cell lines as to propose a mechanism for the progression to malignancy and for a better understanding on this transition. To do this, we elected to study these cells lines and their response at the receptor level as it receives signals from the ECM, signal transduction pathway, and transcriptional level. The latter was previously tested through the use of a plasmid containing the upstream sequence of the mouse ERα promoter region which was spliced to lac-Z gene, the reporter gene. The signaling pathways tested were the PI3/Akt and the MAP kinase pathways. We observed an increased of ERα levels in the presence of lrBM in SCp2 cells. This increase in regulation was also observed and consistent with the Akt signaling pathway. The integrin blocking antibodies α2, α6, and β1 affect the ERα levels by downregulating its expression. This effect may be partly due to the difference in expression of the receptors above, which were lower in SCg6 cells (malignant cell line).

Introduction

Estrogen receptor alpha (ERα) is part of the cell's signals for growth and differentiation and has therefore become an important subject to study in cell cancer and normal cell functioning. In previous studies, it has been shown that the levels of ERα expression decrease in SCp2 cells, phenotipically "normal" mouse mammary epithelial cell line derived from mouse mammary epithelial cell strain CID-9 (Desprez et al. 1993), as they are culture on plastic and that these levels are prevented from decreasing as they are culture in the presence of laminin-rich basement membrane (lrBM) and lactogenic hormones (Novaro et al. 2003) (Figure 1) . It is also known that part of the regulation of the ERα levels is due to collagen-IV and laminin-1, which are specific components of the extracellular matrix, ECM (Novaro et al. 2003). This regulation takes place at the transcriptional level. This led us to examine how the lrBM signaling takes place in regulating the ERα levels on SCp2 cells. Since SCp2 and SCg6 cells share a common derivation, COMMA-1D cells, they can be used as a model to test the progression from normalcy (SCp2 cells) to malignancy (SCg6 cells). From previous results, it has been shown that SCg6 cells, a malignant mouse mammary epithelial cell line that has a common derivation with SCp2 cells, do not regulate ERα levels in the presence of lrBM (Figure 2). Furthermore, SCg6 cells express higher levels of ERα as compared to SCp2 cells (Figure 2). Part of our study is to find out at what level does the regulation due to the lrBM differs from plastic conditions. This is studied at the receptor level of the cell through the use of integrins, at the signal transduction pathway, and as previously shown at the transcriptional level.

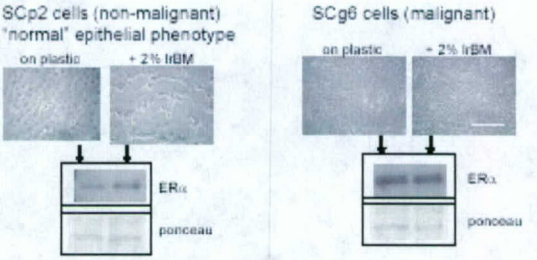
Figure 1: ER-α protein levels are increased following exposure to lrBM



Laminin-rich reconstituted basement membrane (lrBM) added to the medium increases ER-α protein levels in SCp2 cells (Novaro, V Roskelley, CD Bissell, MJ. Collagen-IV and laminin-1 regulate estrogen receptor alpha expression and function in mouse mammary epithelial cells. JCS 2003).

At the receptor level, it has been shown that by using integrin-blocking antibodies for α2, α6, and β1, the ERα levels decrease, showing therefore the blockage of the ECM signaling which prevents the regulation between the cell and the microenvironment surrounding it. The second step then is to find out the effect of blocking-integrins in the absence and presence of lrBM against the two chosen pathways: PI3/Akt kinase and MAPkinase pathways. This is to further dissect the involvement of these pathways in the ECM regulation of ERα since SCg6 cells overexpressed MAPKinase and Akt under both conditions just like the aberrant levels of ERα expression. To test the intracellular transduction pathways, we studied Akt and MAPkinase as some possible elements upstream of the ERα expression involve in the regulation of ERα due to the lrBM. It has been shown that in the presence of lrBM, SCp2 cells besides upregulating ERα expression also upregulate Akt (Figure 3). We have also used LY 294002, LY 294002 inhibits Akt activation by blocking directly P13-K which is upstream of Akt, to further examine its effect on ERα levels in plastic and 2% drip lrBM. From the results, we concluded that Akt is indeed a plausible pathway involve in the ERα regulation by lrBM and that the level of α2, α6, and β1 integrins in SCg6 cells may contribute in part to the lack of responsiveness to the microenvironment.

Figure 2:

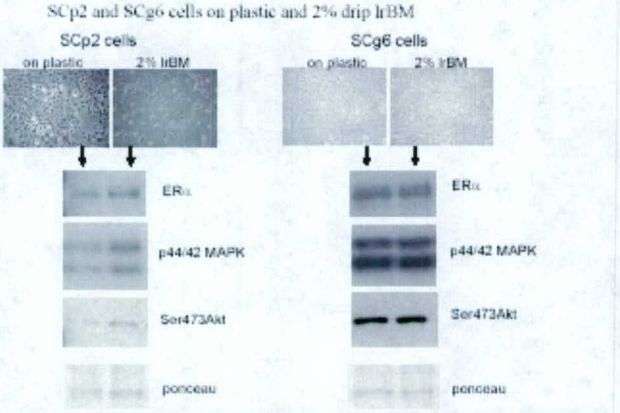


Small cuboidal cell shape changes morphology when lrBM is present (cells cluster together to form round structures). The cells eventually produced β-casein (protein found in milk). The expression levels of ERα are higher in the presence of lrBM (3D) compared to plastic (2D).

Large fibroblast like cell shape is observed in SCg6 cells. They, unlike SCp2 cells, do not respond to lrBM or its components. ERα expression remains the same in the presence or absence of lrBM.

Figure from: Novaro, V., Radisky, D. C., Castro, N. E., Weisz, A., Bissell, M. J. Malignant Mammary Cells Acquire Independence from Extracellular Context for Regulation of Estrogen Receptor α. Clinical Cancer Research. 10: 402-409, 2004.

Figure 3:



SCg6 cells express higher levels of ERα proteins compared to SCp2 cells. SCg6 cells do not respond to lrBM by up-regulating the levels of ERα or by changing its morphology as SCp2 cells do. The levels of MAPK and Akt are also higher in SCg6 cells compared to SCp2. Figure from Dr. Virginia Novaro (Bissell's laboratory)

Materials and Methods

Antibodies and reagents

Anti-E-cadherin monoclonal antibody use was from Tranduction Laboratories (#610182). It is a 120KDa molecule and was diluted 1:1000 in 5% milk in 1xPBS + 0.1% Tween 20.

Anti-ERα antibody use is a polyclonal antibody MC-20 was obtained from Santa Cruz (Santa Cruz, Santa Cruz, CA.)

Integrin-blocking antibodies purchased include α1 (Ha31/8), α2 (HMα2), α6 (GoH3), and β1 (Ha2/5) integrins from PharMingen (San Diego, CA). They were free of sodium

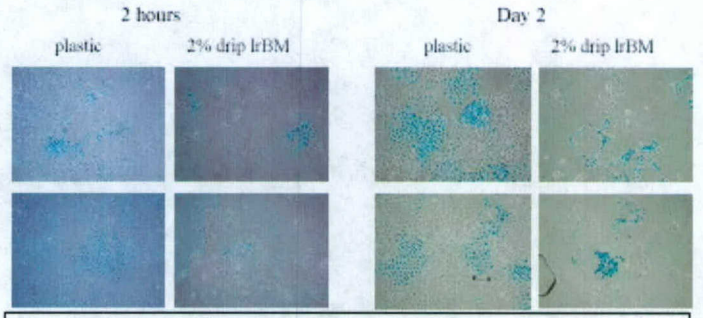
Phospho-Akt (Ser 473) antibody purchased from Cell Signaling Technologies (#9271S)

Anti-PKB alpha/Akt monoclonal antibody was purchased from Tranduction Laboratories (#610837) and was used to test total Akt.

Phospho-p44/42 (T202/4204) monoclonal antibody was purchased from Cell Signaling Technology (#9106S) to test phosphor-MAPkinase.

Figure 4:

X-galactosidase assay, stably transfected SCp2 cells with MERII, 10x



After sorting the cells for β-galactosidase expression, we obtained a higher percentage of cells which were positive. After two hours, ~50% of the cells were blue (express β-galactosidase). After two days of being exposed to the X-galactosidase reagents, a greater percentage of the cells were blue, ~80%. Wild SCp2 cells were also used as a negative control, they did not turned blue (data not shown). The assay may not be sensitive enough for us to determine whether more cells turned blue or if the blueness intensity increases. No significant difference was observed between cells on plastic and 2% drip lrBM.

Total MAPKinase or p44/42 MAPKinase antibody was purchased from Cell Signaling Technology (#9102) and was diluted in 1:1000 with 5%milk (1xPBS+0.1% Tween 20).

Polyclonal anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey) was obtained from Amersham Biosciences (#220836).

Monoclonal anti-mouse IgG, peroxidase linked whole antibody purchased from Amersham Biosciences (NA931V).

Matrigel (laminin-rich reconstituted basement membrane, lrBM) was obtained from Collaborative Biomedical Products (Bedford, MA).

M-PER (Mammalian protein extraction reagent) was used as the lysis buffer for protein extraction and purchased from Pierce (Rockford, IL).

PD 098059 (PD) inhibits MEK1/2. MEK1/2 is a kinase which activates p42 and p44 (MAP Kinase). LY 094002 (LY) inhibits the phosphorylation of PI3 kinase PI3-K in turn activates Akt by phosphorylating it.

For X-galactosidase assay we used several reagents that were provided by Paraic Kenny. The reagents used for the X-galactosidase solution include 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.01% Sodium deoxycholate, 0.02% NP40, 1mg/ml X-gal PBS. The cells were first rinsed with 1X PBS (phosphate-buffered saline). They were then fix with cold 4% paraformaldehyde in 1xPBS for 5 minutes and then wash with cold 1XPBS. The cells were submerged in the X-galactosidase solution and pictures were taken (cells turned blue if positive for β-galactosidase, figure 4). This assay was used to identify and increase the percentage of cells that contain the MER plasmid after cell sorting (reporter gene β-galactosidase) to be subsequently infected with the myr-Akt pWZL retroviral contruct.

Cell Culture

SCp2 is a phenotypically normal cell line derived from mammary epithelial mouse cells (Desprez et. al. 1993). SCp2+MERII is a stably transfected cell culture which was obtained by stably transfecting wild SCp2 cells using Lipofectamine-2000 (Invitrogen) as described by the manufacturer. The plasmid MERII (Mouse Estrogen Receptor II, MERII) was provided by Alessandro Weisz (Cicatiello et. al. 1995). This plasmid has the portion of the mouse estrogen receptor alpha promoter and has been joined to β-galactosidase as the reporter gene. The stably transfected cells (SCp2 +MERII) were selected and the colonies pool together by the addition of neomycin (G418). SCp2+MERII+cA-Akt (constitutive active-Akt or myr-Akt pWZL retroviral contruct) was derived from the already established SCp2+MERII cells. SCp2+MERII cells were infected with the cA-Akt which was prepared by using Phoenix packaging cells. SCp2+cA-Akt were obtained from SCp2 cells and later infected with the cA-Akt. As a control for the cA-Akt infected cells, SCp2+MERII+VC (vector control) were used. The myr-Akt pWZL retroviral construct myrΔ4–129 (Kohn et al., 1998) was given by Richard Roth (Stanford University, Stanford, CA). The transfection of Phoenix packaging cells and production of retroviral stock were prepared according to standard protocols.

Figure 5:

Receptor level expression in SCp2 and SCg6 cells

	SCp2 cells		SCg6 cells	
Integrin level (A.U.)	On plastic	+2%lrBM	On plastic	+2%lrBM
α1	9.5	9.2	18.5	11.6
α2	37.5+/-2	43.6+/-2	27.7+/-0.5	29.4+/-0.3
α6	109+/-2	122.7+/-3	27.9+/-0.3	27.9+/-0.2
β1(n=3)	152+/-12	100+/-10	41.6+/-2	37.3+/-1

Integrin levels differ between SCp2 and SCg6 cells. SCg6 cells have lower levels of α2, α6, and b1 which interact with the microenvironment (ECM components). The difference in integrin levels may explain the abnormal behavior observed in malignancy.

Cells on plastic were directly culture on petri plates with DMEM/F12 medium to which we added 2% serum (fetal bovine serum, FBS), 50 μg/ml gentamycin, 5 μg/ml insulin, 3 μg/ml prolactin and 1 μg/ml hydrocortisone, to allow attachment of the cells to the plate. Cells were cultured in the presence of 2% drip EHS were first plated on petri tissue plates with 2% serum to allow attachment to the surface of the plate. After this, they were rinse with DMEM/F12 medium twice and then added to the plate 2% Matrigel plus the culture media (DMEM/F12).

Western Blots

Follow procedure as indicated by general protocol. The gels used were 10% Tris-Glycine Gel, 1.5mm x 10 well (catalog No. EC6078BOX) which were purchased from Invitrogen life technologies. As washing reagent, we used 1% PBS and 1% PBS + 0.1% Tween 20.

FACS analysis for integrin antibodies

The cells were exposed to lrBM for 2 days (as described above). The cells were harvested using 0.05% trypsin and were resuspended in 2% FBS in DMEM/F12 media.

Eppendorf tubes were use to contain approximately one million cells which were spin at 1000 rpm for 5 minutes. The pellet was washed with FACS buffer (1xPBS, 5% FBS and 0.1% sodium azide) and kept on ice. The cells were incubated with its respective antibody (purchased from Pharmigen) for an hour at four degree and then wash three times with the FACS buffer (the centrifugations after each wash was done at 1000 rpm for 5 minutes at 4 degrees). Incubation of the samples with FITC-conjugated IgG secondary antibody was done for one hour, followed by three washes with FACS buffer. Addition of 2% paraformaldehyde solution to each samples' pellet was done to fix them and mix immediately.

Cell Sorting

We sorted stably transfected SCp2+MERII cells to increase the population of cells that still contain the lac-Z gene. These cells were later use to infect them with the cA-Akt construct. The cells were culture on plastic and harvested using 0.05% trypsin. They were passed through a filter (pore size 0.5μm to 40μm diameter) to allow better separation between the cells for cell sorting. The reagent used was fluorecein di-β-12galactopyranoside substrate reagent to identify β-galactosidase cells. The cell sorting and the FACS analyis was done at the Flow Cytometry Facility in the Cancer Research Laboratory, UC Berkeley. Protocol was done as establish in the manual prepared by Hector Nolla. After the sorting of the cells, they were immediately placed in culture media+ 10%FBS to recover. Different pools depending on β-galactosidase expression were cultured and kept separated.

Results

It has also been shown that the effect produced by laminin-1 and collagen-IV on ERα regulation levels can be disrupted through the use of integrin blocking antibodies α2, α6, and β1 (Novaro et al. 2003) in SCp2 cells. To observe the difference in integrin expression in both cell lines, we use FACS analysis. From it we can appreciate that SCg6 cells have lower levels of α2, α6, and β1 than SCp2 cells (Figure 5). The level of α1 integrin subunit did not varied in SCp2 cells either on plastic or in the presence of lrBM.

To follow up this finding, we proceeded by testing the integrin blocking antibodies and their effect on MAPkinase and Akt. However, we were unable to obtain any significant data due to technical problems with the antibodies used. In the experiment, we tried to test the effect of α1, α2, α6, and β1 integrins on Akt and MAPkinase expression. The use of this antibodies leads to a decrease in the ERα levels in SCp2 cells for the three latter antibodies as shown in previous reports (Novaro et al. 2003). A hypothesis to test is then how would the blockage of the integrins above affect the MAP kinase and Akt levels? We know that Akt and MAP kinase levels are higher in SCg6 cells in the presence and absence of lrBM. No regulation in the expression of ERα is seeing in SCg6 cells (malignant) when compared to SCp2 ("normal") cells. By blocking this, we might observe whether these two pathways or either one of them is involved in the regulation of ERα expression in SCp2 cells. Due to technical difficulties, we could not complete the experiment (blocking antibodies were too old, this was verified by the use of FAK [focal adhesion kinase] as to prove the effect of the blocking antibodies on the integrins, data not shown).

Cell sorting was done on SCp2+MERII cells to increase the efficiency of cells that had the MERII construct and the new infected myr-Akt pWZL retroviral construct.

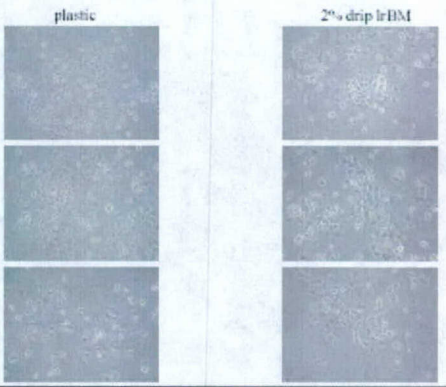
As previously noted, the percentage of cells expressing β-galactosidase was low and through passaging some of the cells have lost the plasmid. Different pools of the sorted cells were isolated. Two different pools from the sorted cells were use to infect with the cA-Akt (myr-Akt pWZL) construct. The cells were culture on plastic and in the presence of 2% drip lrBM. However, the infected cells started to show different features such as lack of cell-cell interaction, distorted shape, and little response to lrBM as compared to SCp2 cells (Figure 6). From the western blots, we observe that the truncated cA-Akt behaves differently from the endogenous Akt (Figure 7). First, a lower molecular weight Akt is seeing (figure 7) and the levels of ERα decrease as compared to SCp2, SCp2+MERII, and SCg6 cells.

Discussion

The unresponsiveness of SCg6 cells to lrBM can be studied at the receptor level specifically α1, α2, α6, and β1 integrins, since the last three integrins seem to respond to lrBM in SCp2 as shown in previous reports (Novaro, et al. 2003). FACS analysis was done in SCp2 and SCg6 cells in the presence of plastic and 2% drip lrBM to compare the expression level of these integrins. SCp2 cells show a greater expression of all the integrins above than SCg6 cells. The difference in expression in SCp2 cells as compared to plastic and 2% drip lrBM starts with α2 and α6 integrin levels which are slightly higher in lrBM whereas α1 levels do not varied and β1 levels were reduced by lrBM. As compared to SCg6 cells, SCp2 cells contain higher levels α2, α6, and β1 integrins (Figure 5). The differences in integrin level in the two cell lines may partly explain the lack of response of the SCg6 cells to lrBM and its independence from its surrounding microenvironment.

Figure 6:

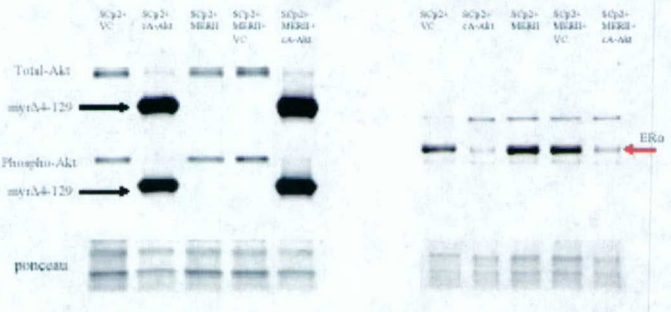
SCp2+MERII+cA-Akt, 10x, day 2



The cells are more dispersed than wild SCp2 cells. Comparing these cells with wild SCp2 cells, we observed that the response to lrBM has changed. After two days in the presence of 2% drip lrBM, SCp2 cells form cell aggregates and cluster together as shown in figure 1. However, the cells infected with constitute active Akt do not respond to lrBM as well as SCp2 cells at day 2. Time course experiment has not been done yet.

Figure 7:

SCp2, SCp2+MERII, SCp2+MERII+VC, SCp2+MERII+cA-Akt western for ERα and phospho-Akt cultured on plastic

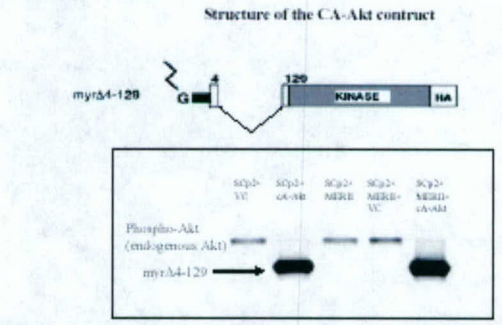


From the figure, we can observe that the truncated form of the cA-Akt plasmid affects the response of ERα levels. The cA-Akt infected cells have lower levels of the cellular Akt and much greater levels of the truncated form (myrΔ4-129). This truncated Akt seems to be affecting the levels of the cellular Akt by decreasing it as well as the ERα levels. Since this truncated form has the opposite behavior from SCg6 cells, a new full Akt construct will be obtained to continue with this study (not yet available).

To study signaling transduction pathway, the pathways chosen for the study include Akt and MAPkinase. These were selected since they both

appear at higher levels in SCg6 cells than its normal counterpart (Figure 3). Then a cA-Akt (constitutively active Akt) construct was used to infect SCp2 and SCp2+MERII cells to obtain a response similar to SCg6 cells. Since SCg6 cells express aberrant levels of Akt, by inserting this constitutively active Akt plasmid, it might lead to the transformation of SCp2 cells to cells more similar to SCg6. After the cells were infected and selected through the use of hygromycin, SCp2+cA-Akt and SCp2+MERII+cA-Akt cells started to show some differences from the wild (SCp2) type and the stably transfected (SCp2+MERII) cells. Figure 3 shows that some of the cells are more dispersed, which may indicate the lack of cell-cell interactions a common characteristic of SCg6 cells. Also some of the cells varied in shape from cuboidal (SCp2 like) to distorted shape. Size of the cells varied as well, some cells have similar size as SCp2 cells while others increase in size. From western blots done in these cells, we observed a decrease in ERα levels. This contradicted part of our expectations which were higher ERα levels as SCg6 cells due to the constitutive active signal of Akt by the plasmid. However, once Akt levels from western blots were obtained, they demonstrate that the Akt molecule from SCp2+cA-Akt and SCp2+MERII+cA-Akt has a lower molecular weight (figure) than the endogenous Akt molecule. This is most likely due to a deletion that is present in the plasmid. The myr-Akt pWZL retroviral construct myrΔ4–129 (Kohn et al., 1998) does not contain the Δ4-129 domain and contains a HA epitope tag at the carboxyl terminus (Figure 8). To continue with the experiment, we recently acquire a plasmid, a gift from Richard Roth, which contains the full length of Akt.

Figure 8:



Myr-Akt pWZL retroviral construct use to infect SCp2 and SCp2+MERII cells. This truncated version of Akt presents a lower molecular weight as shown in the figure. It reproduces a different response in ERα expression. Instead of increasing ERα levels, they decrease which contradicts our findings. To test this again, we have acquired the full length Akt construct (not yet tested).
Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scott, P. H., Lawrence, J. C. Jr., Roth, R. A. Construction and Characterization of a Conditionally Active Version of the Serine/Threonine Kinase Akt. The Journal of Biological Chemistry, 273: 11937-11943, 1998.

Conclusions

Through the use of mouse cell lines, SCp2 and SCg6 cells, we have proposed a model to study and for a better understanding for the transition from normalcy to malignancy. To accomplish this, we use the phenotypically "normal" mouse epithelial cell line known as SCp2 and its malignant counterpart SCg6 cells to dissect this process. It is known that the microenvironment forms part and dictates normal cell functioning. As mentioned above, SCp2 cells when exposed to lrBM regulate the expression of ERα. Since ERα is involve in cell proliferation and differentiation, hence the reason to studied it, has become an important topic. In this case, we studied the effect of the ECM components in ERα regulation and how it differs in SCp2 and SCg6 cells. As previously discussed, SCp2 cells upregulate the levels of ERα in the presence of lrBM. Since the microenvironment interacts with the cells, a receptor mediated interaction and response is expected. This interaction can be block in SCp2 cells through the use of integrin

blocking antibodies for α2, α6, and β1. Furthermore, malignant SCg6 cells express lower levels of integrin than SCp2 cells. The lack of responsiveness in SCg6 cells to the microenvironment could be in part due to the decrease in integrin levels, making SCg6 independent from the ECM signaling. We have also shown that Akt and MAPkinase levels are aberrantly express in SCg6 cells while being control in SCp2 cells. The Akt pathway resulted to be a good candidate in the ERα regulation by the lrBM, since this increase in Akt levels seem to correlate with high ERα expression levels. Through the use of a cA-Akt contruct, we tried to observe the effect of it in SCp2 cells and see whether the infected cells become unresponsive to lrBM like SCg6 cells. However, the construct differs from the endogenous Akt by having a deleted section and shows a different behavior. To confirm the result, we will use the full length Akt construct to continue testing the possible transduction signaling pathways involve in the regulation of ERα by ECM.

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KEY RESEARCH ACCOMPLISHMENTS

- **Karolin Ovrahim** (Bissell laboratory) identified the effects of extracellular matrix molecules on chromosomal DNA structure.
- **Anna Guan** (Kane laboratory) identified ROS responsive genes controlled by transcription factor IIs.
- **Connie Chen** (Bissell laboratory) identified the activity domain of epimorphin, a mammary morphogen involved in branching morphogenesis

- **Grace Marily Cruz** (Nandi laboratory) examined estrogen administration and apoptosis protection during metestrous stage of the estrous cycle.

- **Deepti Nahar** (Bissell laboratory) used microarray techniques to profile a human breast cancer progression system.

- **Karla Galvez** (Bissell laboratory) explored the relationships between physical alteration of mammary epithelial cell morphology (by stretching on a flexible substratum) and cell differentiation.

- **Irving Salmeron** (Barcellos-Hoff laboratory) demonstrated that human fibroblasts show an increased p53 response to decreased TGFβ levels.

- **Nancy Castro** (Bissell laboratory) examined the effects of microenvironment on estrogen receptor expression.

REPORTABLE OUTCOMES

Anna Guan and **Grace Marily Cruz** graduated with B.S. degreez, and used their research in their honors theses. **Connie Chen, Grace Marily Cruz, and Anna Guan** will present their work in a poster at the 2005 Era of Hope meeting. As of yet, no publications or patents have derived from this work.

CONCLUSIONS

The second year of research trainees was just as successful as the first, and most of the summer trainees stayed on in their training labs for the remainder of the academic year, either as research associates or on a volunteer basis. Several manuscripts, currently in preparation, contain experimental results produced by the undergraduate trainees. The third year will benefit from organization principles learned from the first two years